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Award Number: DAMD17-02-1-0183

TITLE: Molecular Biology and Prevention of Endometrial Cancer

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REPORT DATE: July 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20050315 031

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)**2. REPORT DATE**

July 2004

3. REPORT TYPE AND DATES COVERED

Annual (17 Jun 2003 - 16 Jun 2004)

4. TITLE AND SUBTITLE

Molecular Biology and Prevention of Endometrial Cancer

5. FUNDING NUMBERS

DAMD17-02-1-0183

6. AUTHOR(S)

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Advancement of Military Medicine
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REPORT NUMBER****9. SPONSORING / MONITORING****AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**

Objective: To increase our understanding of the molecular aberrations associated with endometrial carcinogenesis and the biologic mechanisms underlying the protective effect of oral contraceptive therapy. Methods: 1) Oligonucleotide microarray analysis was performed on a panel of endometrial cancers. 2) A subset of adenocarcinoma cases from the International DES Registry was analyzed for MSI and the poor quality of the DNA from many of these cases prohibited an adequate analysis of the entire set. The cases are currently being evaluated for PTEN expression. 3) We are in the process of analyzing the endometrium from cynomolgus macaques that were exposed to long term progestins as part of the three year randomized controlled trial. 4) A clinical trial comparing progestin versus placebo is underway that will facilitate investigation of the effects of progestin exposure on the endometrial lining.

Results: 1) Different histological types of endometrial cancer have unique genomic expression patterns. 2) Endometrioid endometrial cancers with microsatellite instability have a genomic expression pattern that is different from those microsatellite stability.

14. SUBJECT TERMS

endometrial cancer, hormone, prevention

15. NUMBER OF PAGES

12

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Endometrial cancer is the most common type of gynecologic cancer in the United States and was estimated by the American Cancer Society to have been newly diagnosed among 36,000 American women in the year 2000 and lead to approximately 6500 cancer related deaths (1). Approximately 25% of all endometrial cancers occur in premenopausal women (2). Major advances in our understanding and treatment of endometrial cancer have occurred over the past decade, yet the frequency of this cancer in the general population has not been altered appreciably. Despite the known protective effect of oral contraceptives, little has been learned regarding the underlying mechanism. We believe that an understanding of the molecular profiles of endometrial cancers and the molecular events underlying the protective effect of oral contraceptives against endometrial cancer could facilitate the development of effective chemopreventives and significantly decrease the incidence of endometrial cancer in women.

BODY

Aim 1: To characterize and compare the molecular profiles of Type I endometrioid endometrial cancers, which often develop in an estrogen milieu, to that of Type II endometrial cancers. In addition, we will use microarray to examine the molecular changes in the endometrium associated with progestin exposure in order to gain insight into the biologic mechanism underlying the chemopreventive effect of the oral contraceptive pill (OCP).

- 80 cases of endometrial carcinoma (56 endometrioid and 24 papillary serous) were examined in addition to 10 cases of dissected normal endometrium from age-matched patients. Total RNA from each sample was analyzed using the Affymetrix HG133A and HG133B GeneChip set. Data analysis was performed using unsupervised multidimensional scaling (MDS), binary class comparison, and hierarchical clustering. A subset of individual gene expressions was quantified and validated using real-time PCR. Multidimensional scaling revealed that the expression profiles of endometrioid and papillary serous carcinomas as well as normal endometrium were different (Figure 1A). In a supervised comparison, 160 of these genes were found to be differentially expressed by at least at 2-fold ($p < 0.001$) (Figure 1B). We further analyzed these two groups of cancers using class prediction models. The nearest centroid classifier predicted 91% of the arrays correctly with a p-value of 0.001. The accuracy of this prediction was verified using 4 other classifiers including support vector machine, compound covariate predictor, and linear discriminant analysis, which also correctly classified the samples in 88-89% of the arrays, further validating the data. Examples of genes that were notably associated with a >2 -fold PS/E expression ratio included *IGF2*, *PTGS1(COX1)*, and *p16* while genes with a >2 -fold E/PS expression ratio included *TFF3*, *FOXA2*, and *MSX2*. These data confirms the hypothesis that distinct patterns of gene expression characterize various histologic types of uterine cancer. An understanding of the molecular heterogeneity of various histologic types of endometrial cancer has the potential to lead to better individualization of treatment in the future.
- A subset of 24 endometrioid endometrial cancer cases was analyzed for microsatellite instability (MSI) using 5 markers (i.e. BAT25, BAT26, D2S123, D5S346, and D17S250). Cancers were designated as having MSI when at least 2 of the 5 markers exhibited differences in allele size as compared to the patients matched normal DNA. The other types (papillary serous, clear cell, etc) were not analyzed since MSI is uncommon in these histologic types. Total RNA from each sample was analyzed using the Affymetrix HG133A and HG133B GeneChip set. Data analysis was performed using unsupervised principle component analysis (PCA), binary class comparison, and hierarchical clustering. A subset of individual gene expressions was quantified and validated using

real-time PCR. Unsupervised PCA of the expression data from these cases indicated two distinct groupings of cancers based on the MSI phenotype. We identified 392 genes that differed between MSI and microsatellite stable (MSS) cases at high significance ($P=0.001$) with 109 of these genes different by at least 2 fold. Both the hMLH1 and the KIAA0766 gene were distinguishing transcripts on the differentially expressed gene list. Since these genes share the same CpG island that is hypermethylated in many MSI cancers, we have identified an important internal control lending validity to our results. A leave-one-out prediction model using these 392 genes revealed that the class prediction of MSI status was 92%. These data identify distinct expression profiles in MSS and MSI endometrial cancers. These profiles indicate that cancers with MSI develop in part by differing mechanisms than do their similar MSS counterparts and these 2 distinct groupings are more likely to have implications affecting therapy and prevention.

- Since the rate of PTEN alteration was found to be approximately 95%, an analysis of the array data based on PTEN status was not felt to have an adequate number cases with wild type PTEN to enable an accurate analysis. Perhaps a future microarray study aimed at an analysis of PTEN in very early stage endometrial cancers and precancerous endometrial conditions would facilitate an expression profile associated with alterations in PTEN.

Pending

- The short-term effects of progestins gene expression in the endometrium will be evaluated using uterine specimens collected from patients enrolled in a double-blinded prospective randomized trial. The initiation of this trial had been delayed secondary to multiple revisions requested by multiple IRB committees. However, the protocol recently has been approved and the study is now being initiated at both Walter Reed and Evanston Northwestern. The tissue collected as part of this trial will also be used for microarray analysis of microdissected endometrium samples. A no cost extension has been approved for completion of this research.

Aim 2: To analyze vaginal and cervical adenocarcinomas, that have arisen in women exposed to DES in-utero for methylation and mutation of PTEN and MLH1 in order to determine if estrogen induces genetic alterations in these tumors characteristic of Type I endometrioid carcinomas.

Although a pilot study aimed at an analysis of MSI in 7 cases from the International DES Registry was successful with repetitive attempts at DNA amplification (Appendix 6), the analysis of the entire set was not successful presumably secondary to the quality of the DNA which reflects the old age of the specimens and the various methods that were used in their preservation. Less than 50% of the samples amplified at any one of the markers making the data inadequate for designation of MSI status.

Our current intent is to evaluate the cases for PTEN immunostaining with the intent to evaluate for methylation of the PTEN promoter in cases with altered PTEN expression. The methodology for PTEN staining using human cancer has recently been optimized in preparation for analysis of the samples that are being evaluated as part of Aim 4. Because we exhausted supply of tissue provided to us by the Transplacental Tumor Registry in attempting to complete the MSI analysis, we are currently in communication with the Registry to obtain additional tissue specimens. Our intent is to complete the PTEN staining of these human tissue concurrently with the staining of tissues collected as part of the prospective randomized comparison of progestin versus placebo in Aim 4. A no cost extension has been approved for completion of this research.

Aim 3: Using data from the Centers for Disease Control Cancer and Steroid Hormone Study, we will determine if the protective effect of OCP's against endometrial cancer are impacted by the progestin or estrogen potency of OCP formulations.

Completed year 1 of the grant. Manuscript is being reviewed by the Journal of the American Medical Association

Aim 4: To test the hypothesis that the oral contraceptives and hormone replacement therapy progestins provide a chemoprotective effect against endometrial cancer through induction of apoptosis, PTEN, and TGF-beta in the endometrium.

Epidemiological studies have demonstrated that OCP use lowers the risk of subsequent endometrial and ovarian cancer. Although the biologic mechanism(s) underlying the protective effect of OCP's on the risk of both of these cancers have not been well defined, there is evidence to suggest that biologic effects related to the progestin component may underlie the cancer preventive effects of the OCP. Recent studies have reported the progestin-mediated activation of apoptosis in endometrial cancer cell lines and endometrial hyperplasias. The finding that progestin activates the apoptosis pathway in endometrial cells raises the possibility that this may be a major mechanism underlying the therapeutic effect of progestins against endometrial hyperplasia. Similarly, our group has found that progestins markedly activate both apoptosis and TGF-beta expression in the ovarian epithelium leading to the hypothesis that progestins may act as chemopreventives for ovarian cancer (see preliminary data below). It is interesting that tumors arising from the ovary and endometrium share common epidemiological risk factors, and that both the endometrium and ovarian surface epithelium share a common embryological precursor. It is thus plausible that progestins activate similar molecular pathways relevant to cancer prevention in both of these organ sites. Recent evidence suggests that expression *PTEN* appears to be upregulated in the secretory phase of the menstrual cycle. It is plausible that the chemopreventive effects of OCP's are mediated through overexpressed *PTEN* with resultant suppression of cell cycle progression and activation of apoptosis in endometrial cells.

- The long term effects of progestins on apoptosis as well as the expression of PTEN and TGF-b in the endometrium were to be evaluated using uterine specimens from 210 cynomolgus macaques (80 premenopausal and 130 postmenopausal) previously part of a three-year randomized trial designed to evaluate the effects of the combination oral contraceptive pill and hormone replacement therapy on reproductive organs. The assay conditions for the analysis of these samples has been optimized for the TGF- β and the preparation of these slides is underway at Evanston Northwestern. The staining of the macaque slides for PTEN was completed but interpretation of the set of slides that was stained in 3 separate runs was found on review of the slides to be less than optimal for interpretation, due to inconsistencies in the staining intensity of replicate samples and the equivocal staining and loss of PTEN staining in the macaque samples. Our suspicion is that the inconsistencies in staining may reflect the human PTEN antibody being used on the macaque tissue. Several samples have been sent to Dr Charis Eng at Ohio State University for independent immunohistochemical analysis. Dr Eng's lab has performed immunohistochemical staining for several sentinel papers involving the PTEN tumor suppressor gene and cancer. If Dr Eng verifies that it has been problems related to our methods, we plan to restrain the cases using her recommended algorithm. If her lab confirms limitations of the commercially available PTEN antibody in staining macaque tissues, then we will abandon this aspect of Aim 4. Dr Cline has completed the staining for

apoptosis at Wake Forest University and preliminary review of the slides has confirmed adequate staining for interpretation. The formal review of these slides and interpretation of data is currently underway. A no cost extension has been approved for completion of the research related to the long-term effects of progestin on the endometrial lining.

- The short-term effects of progestins on apoptosis as well as the expression of *PTEN* and TGF- β in the endometrium will be evaluated using uterine specimens collected from patients enrolled in a double-blinded prospective randomized trial. The initiation of this trial has been delayed secondary to multiple revisions requested by multiple IRB committees. However, the protocol recently has been completed and the study is now underway at both Walter Reed and Evanston Northwestern. The endometrial specimens are currently being collected and have not undergone analysis. The tissue collected as part of this trial will also be used in part 2 of Aim 1, which will involve a microarray analysis of microdissected endometrium samples. A no cost extension has been approved for completion of this research

KEY RESEARCH ACCOMPLISHMENTS

- Identified additional genes that are differentially expressed in endometrial cancer vs. normal endometria.
- Identified genes that are differentially expressed between endometrioid and papillary serous endometrial carcinoma and determined that histology can be predicted on the basis of gene expression in approximately 90% of cases
- Confirmed that microsatellite stable endometrial cancers have unique gene expression profiles compared to those with microsatellite instability

REPORTABLE OUTCOMES

Research

Abstracts

- Maxwell GL. 2004. Progestin and estrogen potency of combination oral contraceptives and endometrial cancer risk. *Gynecologic Oncology* 2004;92:394, Abstract 3.
- Maxwell GL, Chandramouli G, Dainty L, Litzi T, Berchuck A, Barrett JC, Risinger J: Microarray analysis of different histologic types of uterine cancer reveals unique expression profiles. *Gynecologic Oncology* 2004;92:405, Abstract 27.
- Risinger JI, Maxwell GL, Chandramouli G, Dainty L, Litzi T, Assad U, Berchuck A, Barrett JC: Gene Expression Profiling of Microsatellite Unstable (MSI-H) and Microsatellite Stable (MSS) endometrial cancers indicates separate gene expression profiles. *Gynecologic Oncology* 2004;92:440, Abstract 104.

Presentations

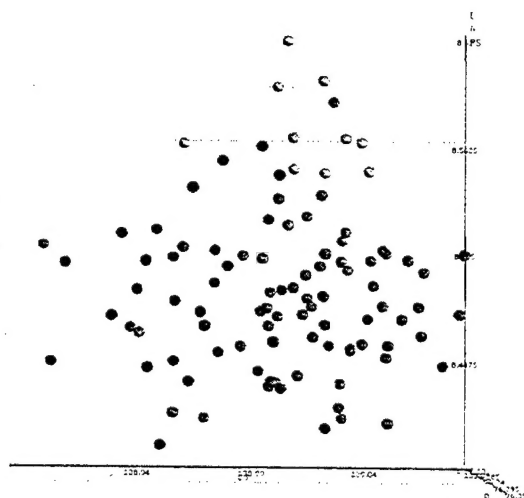
- Maxwell GL: Microarray analysis of different histologic types of uterine cancer reveals unique expression profiles. Plenary session, Society of Gynecologic Oncology, 2004, San Diego.
- Maxwell GL. 2004. Progestin and estrogen potency of combination oral contraceptives and endometrial cancer risk. Plenary session, Society of Gynecologic Oncology, 2004, San Diego.
- Risinger JI, Maxwell GL, Chandramouli G, Dainty L, Litzi T, Assad U, Berchuck A, Barrett JC: Gene Expression Profiling of Microsatellite Unstable (MSI-H) and Microsatellite Stable (MSS) endometrial cancers indicates separate gene expression profiles. Poster session, Society of Gynecologic Oncology, 2004, San Diego.

CONCLUSIONS

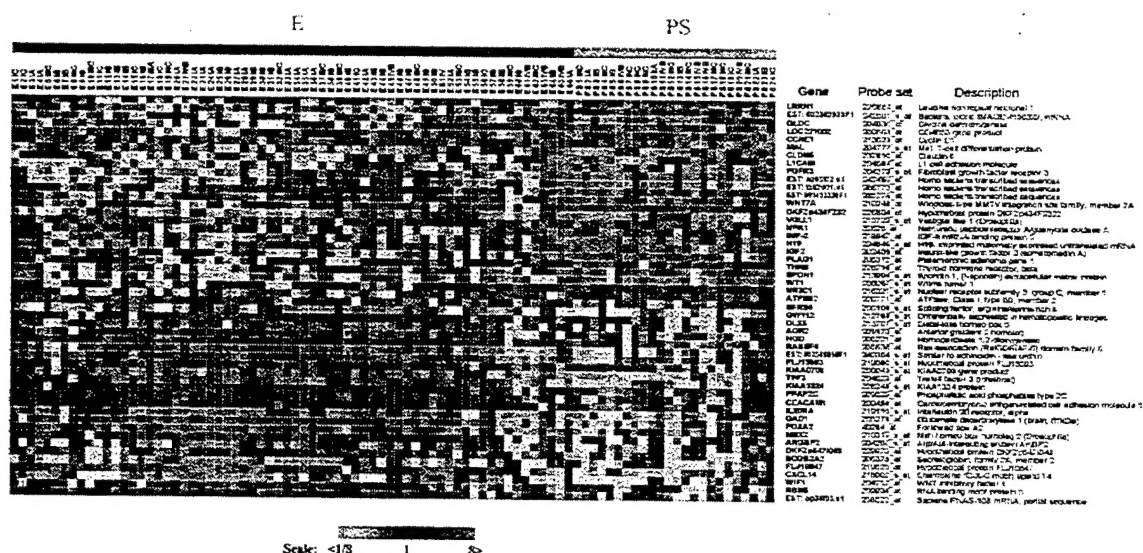
Different histological types of cancer have genomic expression patterns that reflect unique pathways of carcinogenesis. Likewise, cancers characterized by microsatellite instability result in the expression of genes most likely to be affected by alterations in mismatch repair. As we improve our understanding of the alterations that accompany endometrial carcinogenesis, it is likely that future chemopreventives may be developed for several types of endometrial cancer, each of which develops by specific pathways.

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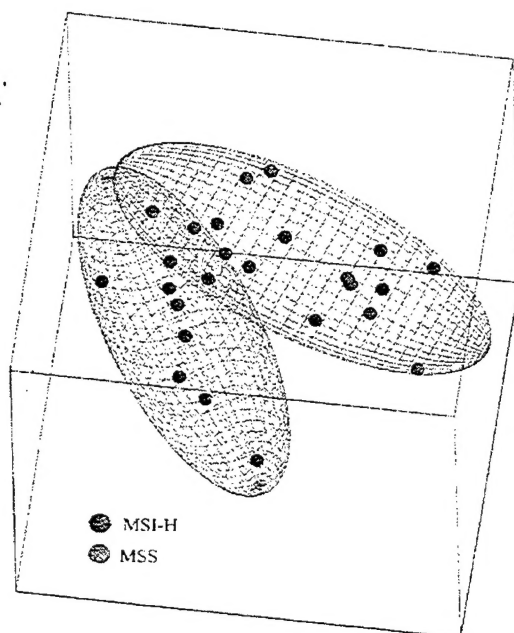


A: A: Multidimensional scaling based on the overall gene expression in endometrioid (red), papillary serous (blue), and normal endometrium (green). **B:** Differentially expressed genes between 66 endometrioid carcinomas and 24 papillary serous carcinomas. 25 most upregulated and 25 most down regulated genes at $p < 0.001$. Each sample in the heat-map is labeled histology, stage of disease and coded tumor number.



B: A: Unsupervised analysis using principal component analysis based on the overall gene expression in microsatellite unstable and microsatellite stable endometrioid endometrial carcinoma.

Appendix 2: Unsupervised analysis of microsatellite unstable versus microsatellite stable endometrioid endometrial cancer



Appendix 3: Supervised analysis identifying genes differentially expressed between microsatellite unstable and microsatellite stable endometrial cancers

